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PRINCIPAL INVESTIGATOR: Peter Maxim, PhD

CONTRACTING ORGANIZATION: The Leland Stanford Junior University
Stanford, CA 94305

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14. ABSTRACT: In this project we propose to experimentally characterize the radiobiological effectiveness of delivery times in the sub-second range using very high energy electrons (VHEE) on lung cancer cells in-vitro and in-vivo. To carry out the proposed project, we will use established procedures such as clonogenic cell survival essays for the *in vitro* studies to study the dose rate effects, and *in vivo* tumor growth delay assays to study the RBE of VHEE. For this purpose, we have established a unique multidisciplinary collaboration between Stanford University Department of Radiation Oncology and the Accelerator Research Division at Stanford Linear Accelerator Center (SLAC) National Accelerator Laboratory. Our in-vitro studies have demonstrated a statistically significant higher cell kill with fast irradiation times compared to conventional irradiation times. We have not observed a difference in cell survival between 60 MeV vs 120 MeV irradiations or between VHEE and photons

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REPORT OF INVENTIONS AND SUBCONTRACTS

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SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) *(Last, First, Middle Initial)* a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS *(X)* d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER *(X)* e.	
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None	None	None						

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(b) NAME OF EMPLOYER N/A	(b) NAME OF EMPLOYER		
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SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS *(Include ZIP Code)* b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES *(YYYYMMDD)* f.	
			(1) CLAUSE NUMBER	(2) DATE *(YYYYMM)*		(1) AWARD	(2) ESTIMATED COMPLETION
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I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL *(Last, First, Middle Initial)*	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Peter Maxim, PhD	Assistant Professor of Radiation Physics	P. Max	20150218

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Contract number: W81XWH-14-1-0014

Title: Very High Dose-Rate Radiobiology and Radiation Therapy for Lung Cancer

Principal Investigator: Peter G Maxim

Introduction:

In the U.S., lung cancer is the leading cause of cancer death. Worldwide, the global burden of lung cancer is increasing dramatically, and the number of patients who could benefit from RT far exceeds its availability. Radiation therapy (RT) has long been a core modality in curative-intent therapy for lung cancer, mainly in locally advanced disease however, cure rates have historically been suboptimal. Because of major technological advances in recent years, specifically highly conformal, accurate, and dose-intensive RT with image-guided target definition and delivery verification, RT is rapidly becoming a component of potentially curative therapy for properly selected patients in every stage of lung cancer. The most dramatic example is stereotactic ablative radiotherapy (SABR)/ stereotactic body radiation therapy (SBRT), highly focused and accurate radiation delivered in a few large doses rather than the conventional fractionated treatments using small doses, for early stage lung cancer in patients with high surgical risk or contraindications.

One fundamental remaining barrier to precise, accurate, highly conformal radiation therapy is patient, tumor, and organ motion from many sources including musculoskeletal, breathing, cardiac, organ filling, peristalsis, etc. that occurs during treatment delivery. Therefore, tremendous effort in our field has been devoted to developing motion management strategies in order to suppress, control, or compensate for motion. **A fundamentally different approach to managing motion is to deliver the treatment so rapidly that there is no time for significant motion, thus increasing the precision and accuracy of lung SABR/SBRT.**

Objective: We propose to develop a new type of RT system for early stage lung cancer using rapidly scanned beams from many directions through electromagnetic steering with no mechanical moving parts, referred to as **pluridirectional high-energy agile scanning electron radiotherapy (PHASER)**. In this project we propose to experimentally characterize the radiobiological effectiveness of delivery times in the sub-second range using VHEE. For this purpose, we have established a unique multidisciplinary collaboration between Stanford University Department of Radiation Oncology and the Accelerator Research Division at Stanford Linear Accelerator Center (SLAC) National Accelerator Laboratory, with the following goals:

Overall Project:

Task 1 - Specific Aim 1: To characterize the impact of dose delivery time on tumor control *in vitro* and *in vivo* (months 1-5)

Task 1a: Purchase human lung cancer cells A549 and human lung squamous cell carcinoma line SK- MES-1 from ATCC (month 1).

Four different cell lines of varying radiosensitivity were acquired for the ***in vitro*** experiments: A549 (lung adenocarcinoma), HCT116 (colon cancer), HT-1080 (fibrosarcoma), and U-87 MG (glioblastoma). We have added additional non-lung cancer cell lines, to determine whether the impact of the dose delivery time is a universal phenomenon that is not only restricted to lung cancer.

Task 1b: Growing tumor cells will be plated into Petri dishes and exposed to very high dose

rate VHEE radiation and 10MV photon irradiation. Following irradiation (as described in the Project Narrative), cells will be were incubated for 10–14 days for colony formation. Colonies will be fixed, stained and counted (months 2-5).

Cells were irradiated using two different types of particles, photons or electrons, and different energies. They were exposed to different doses during different irradiation times to evaluate the effect of dose delivery time on cell survival. The ***in vitro*** experiments are summarized in table 1. All irradiations were done in triplicate.

The different cell lines were all irradiated under "incubator conditions" in a mini-incubator shown in Figure 1 to maintain physiologic relevant conditions:

- Adherent monolayers grown in T25 flasks
- 37° C, pH 7.4, 5% CO_2/buffer system (maintained in the mini-incubator)

Table 1. Summary of the ***in vitro*** experiments performed with clinical LINACs.

Particle	Energy	Irradiated cell lines	Dose (Gy)	Irradiation times
Photons	6 MV	A549, HCT116, HT-1080 and U-87 MG	2, 6, 10, 12	30 s and 30 min
Electrons	9 MeV	A549, HCT116, HT-1080 and U-87 MG	10	10 s, 30 s, 3 min, and 30 min
	60 MeV (SLAC) 120 MeV (SLAC)	A549, HCT116	2, 6, 10, 12	15 min, 20 min

For the photon irradiations, we used a Varian ® TRILOGY linear accelerator (LINAC), in stereotactic mode (1000 MU/min) and a reduced source to surface distance (SSD) of 72 cm to achieve the highest possible dose rate with this particular clinical LINAC (~ 21 Gy/min for the TRILOGY) (Figure 1).

Conventional energy electron irradiations were done using a clinical Varian ® Truebeam LINAC in high dose rate total skin mode (HDTSe=2500 MU/min) and a reduced SSD of 64 cm to obtain again the highest possible dose rate (~ 67 Gy/min). In Figure 2, the experimental setup used for these irradiations is shown.

Conventional energy photon and electron irradiations performed with clinical LINACs took place at the Cancer Center in the Department of Radiation Oncology (School of Medicine, Stanford University).

Dosimetry was performed before all the irradiations using radiochromic film Gafchromic ® model EBT2. Profiles for both types of particles showed a flatness of 3%.

After the irradiations, cells were plated and grown for two weeks to evaluate clonogenic cell

survival. Colonies were then fixed, stained, and counted. Figure 3 shows an example of colony formation.

Task 1c: Inoculate tumors subcutaneously in the back of each mouse and monitor 3 times weekly by BLI and by caliper measurements. Irradiation experiments will be performed when the tumors have reached a volume of approximately 150 mm^3 at 2-3 weeks after tumor inoculation (month 2-5).

Before evaluating tumor control by irradiation ***in vivo,*** we examined the effect of dose delivery time on normal tissue. For that purpose, the brains of male C57BL/6J mice (8-10 weeks) were irradiated using 10 MV photon beams from a clinical Varian ® Truebeam LINAC in Flattening Filter Free mode (FFF=2400 MU/min) and a reduced SSD to obtain the highest possible dose rate.

Mice were placed on top of an acrylic slab that permitted the positioning of the animals near the beam exit (Figure 4a). Animals were anesthetized with isoflurane gas (2% in 98% O_2) via inhalation using a rodent nose cone.

Mega voltage images of the mice were acquired before the irradiations to determine the target in an accurate manner (Figure 4b).

The corresponding radiochromic film dosimetry was done before and during irradiations (Figure 4c) to verify that the dose delivered out of clinical reference conditions was correct.

Two time frames have been evaluated: 20 Gy delivered in 33 s as compared to 20 Gy in 15 min. Table 2 summarizes the ***in vivo*** irradiations that were performed with conventional clinical photon beams.

Determination of patterns of inflammation at 6 months post-irradiation will be done using immunohistochemistry (data pending).
The tumor irradiation studies at SLAC will be conducted once the normal tissue response studies are concluded.

Table 2. Summary of the ***in vivo*** experiments performed with a clinical LINAC.

Particle	Energy	Dose (Gy)	Irradiation times	Number of irradiated mice
Photons	10 MV	20	33 s	9
			15 min	9

<u>Task 2 - Specific Aim 2:</u> To characterize the RBE of VHEE vs. MV photons for tumor control *in vitro* and *in vivo* (months 6-10)

Task 2a: ***In vitro clonogenic survival assay:*** In addition to the 10 MV photon and 100 MeV electron irradiation experiments described above, 50 and 75 MeV electron doses of 5, 10, 15, and 20 Gy each will be administered to cell suspensions over 5 min, and clonogenic survival will be determined using the same procedures as above. This will provide a set of matched experiments (at 5, 10, 15, and 20 Gy, delivered over 5 min) using 10 MV photons and 50, 75, and 100 MeV electrons to quantify the differences in clonogenic survival owing to radiation quality (months 6-10).

The very high energy (60 MeV and 120 MeV) electron irradiations were performed at the Next Linear Collider Test Accelerator (NLCTA) facility located at the SLAC National Accelerator Laboratory. This two energies were the only stable energies operating at the facility, thus we were not able to achieve a stable 75MeV beam. The electron beam, produced by an S-band RF photoinjector, is further accelerated by two high-gradient X-band RF accelerating structures and is transported approximately 25 meters to the experimental station inside a beam line. There are several quadrupole and dipole magnets to transport and shape the beam and diagnostics to monitor beam energy, energy spread, charge, beam size, and beam position. The NCLTA beam line was modified to accommodate the experimental setup.

For the high energy electron irradiations, we placed the mini-incubator on top of a motorized scanning stage and in front of the beam line. Inside the incubator, we placed the flasks containing the cells on top of an acrylic slab that was attached to a rotation motor. The motor allowed the irradiation of the flasks from two sides. Every flask was irradiated from one side, rotated, and irradiated from the other side to obtain a more homogeneous dose distribution. Figure 5 shows the experimental setup used for the high energy electron irradiations.

Table 3 shows a summary of the ***in vitro*** experiments that were performed using VHEE. All the irradiations were done in triplicate.

Dosimetry was performed before all irradiations using radiochromic film Gafchromic ® model EBT2. Figure 6 shows an example of the dose distributions obtained from the dosimetry. Profiles showed a flatness of 5%.

Again, after the irradiations, cells were plated and incubated for two weeks to evaluate colony formation. Colonies were then fixed, stained, and counted.

Table 3. Summary of the ***in vitro*** experiments using VHEE.

Particle	Energy	Irradiated cell lines	Dose (Gy)	Irradiation times
Electrons	60 MeV 120 MeV	A549, HCT-116,	2, 6, 10, 12	15 min, 20 min

Task 2b ***In vivo tumor growth delay:*** In addition to the 10 MV photon and 100 MeV electron irradiation experiments described above, 50 and 75 MeV electron doses of 15 and 30 Gy each will be administered to SQ tumors over 5 min, and tumor growth delay will

be determined using the same procedures as above. This will provide a set of matched experiments (at 15 and 30 Gy, delivered over 5 min) using 10 MV photons and 50, 75, and 100 MeV electrons to quantify the differences in tumor growth delay owing to radiation quality (months 6-10).

Due to long delays with the NLCTA beam line modification and difficulties creating a large enough VHEE field to irradiate the entire tumor we have first focused on characterizing the dose rate effect on normal tissue.
For the purpose of evaluating the effect of dose delivery time on normal tissue sparing, we have irradiated the lung tissue of male C57BL/6J mice (8-10 weeks) at the NLCTA facility using VHEE of 120 MeV.

Computed tomography (CT) images were acquired prior to irradiations to identify, from anatomic landmarks, the position of the right lung in mice (Figure 7). With this information, it was possible to position the mice in front of the beamline to irradiate a partial volume of the right lung.

Animals were anesthetized with isoflurane gas (2% in 98% O_2) using an anesthesia box. The box was placed on top of a scanning stage and in front of the beam line (Figure 8). Mice were positioned using a laser that corresponded to the beam incidence (Figure 9a). Dosimetry using radiochromic films was done before and during the irradiations for every animal (Figure 9b).

Two time frames have been evaluated: 1 s for the fast dose delivery and ~ 10 min for the conventional dose delivery. Doses ranging from 18 to 60 Gy have been considered. Table 4 summarizes the ***in vivo*** irradiations performed at NLCTA using VHEE.

Table 4. Summary of the ***in vivo*** experiments performed with clinical LINACs.

Particle	Energy	Irradiation times	Dose (Gy)	Number of irradiated mice
Electrons	120 MeV	1 s	60	3
			40	3
			18	3
		12 min	60	3
		8 min	40	3
		3 min	18	3

For these experiments, we will acquire bi-weekly CT images of the mice to monitor fibrosis up to a 6 month period following irradiation. We will also be performing immunohistochemistry to characterize apoptosis and fibrosis patterns to determine the normal tissue sparing properties of VHEE irradiations.

Task 3: Data analysis and submission for publication in peer reviewed journal (months 11-12).

Figure 10 shows the survival curves obtained from the photon irradiations performed using a conventional clinical LINAC. The clonogenic survival analysis corresponding to clinical electron beam irradiations is shown in Figure 11. Figure 12 shows the survival curves obtained from the VHEE irradiations. In all cases we have demonstrated a higher cell kill with the faster (30 seconds) irradiations compared to slow/conventional irradiation times (30 minutes). As shown in Figure 12, we did not observe a difference in survival curves between 60MeV and 120MeV VHEE. Based on published literature the effect of 60MeV and conventional energy photon is anticipated to be the same and this is supported by our data.

The objective of the ***in vivo*** experiments was to evaluate the effect of dose delivery time on normal tissue. This has been done by observing late radiation-induced effects on normal tissue, such as fibrosis in the lung. These late effects are manifested several months after irradiation (up to 6 months). For this reason, the results from the ***in vivo*** experiments are still being acquired and processed. Once the data of the dose rate effects on normal tissue are characterized and we overcome the difficulty of creating a larger VHEE beam, we will start with the proposed tumor response experiments.

Key Research Accomplishments:

- We have demonstrated for several cancer cell lines in vitro that faster irradiations cause a higher cell kill that conventional slow irradiation times.
- We have not observed a difference in cell survival between 60 MeV vs 120 MeV irradiations or between VHEE and photons.
- In addition to the planned tumor control experiments, we are analyzing the dose rate effects on normal tissues as well.
- We have presented our findings at national and international conferences and acknowledged DoD support (please see below).
- A manuscript detailing our studies is in preparation.

Reportable Outcomes:

The following abstracts have been selected for POSTER presentation:

Abstracts

1. Rafat M, Bazalova M, Palma BA, Kozak MM, Jiang D, Dunning M, McCormick DJ, Nelson JL, Hemsing E, Lartey FM, Graves EE, Koong AC, Maxim PG, Loo BW. Impact of Very Rapid Irradiation on Clonogenic Survival. American Society for Radiation Oncology; 09/2014.

Oral Presentations

1. Rafat M, Bazalova M, Palma BA, Kozak MM, Jiang D, Dunning M, McCormick DJ, Nelson JL, Hemsing E, Lartey FM, Graves EE, Koong AC, Maxim PG, Loo BW. Radiobiological Advantage of Very Rapid Irradiation. American Association of Physicists in Medicine, Annual Meeting; 07/2014 (Oral Presentation).

2. Rafat M, Bazalova M, Lartey FM, Graves EE, Maxim PG, Loo BW. Biological Impact of Very Rapid Irradiation. Canceropole Grand Ouest, The Future of Radiation Oncology: Imaging, Dosimetry, Biology & Therapy Workshop; 09/2013 (Oral Presentation).

Impact of Very Rapid Irradiation on Clonogenic Survival

M. Rafat,[1] M. Bazalova,[1] B.A. Palma,[1] M.M. Kozak,[1] D. Jiang,[1] M. Dunning,[2] D.J. McCormick,[2] J.L. Nelson,[2] E. Hemsing,[2] F.M. Lartey,[1] E.E. Graves,[1] A.C. Koong,[1] P.G. Maxim,[1] and B.W. Loo[1]
[1]Stanford University, Stanford, CA, [2]SLAC National Accelerator Laboratory, Menlo Park, CA

Purpose/Objective(s): We characterized the effect of very rapid dose delivery as compared to conventional therapeutic irradiation times on clonogenic cell survival. This work represents the first step toward elucidating the radiobiology of radiation therapy (RT) sufficiently fast to eliminate the complications of tumor motion during treatment.

Materials/Methods: We used a Varian Trilogy linear accelerator to deliver doses up to 14 Gy using a 6 MV photon beam. We irradiated four cancer cell lines (A549, HCT-116, HT-1080, U87-MG) in times ranging from 30 sec to 30 min. We also used a Varian TrueBeam linear accelerator to deliver 9 MeV electrons at 10 Gy in 10 s to 30 min to determine the effect of irradiation time on cell survival. We then evaluated the effect of using 60 and 120 MeV electrons on cell survival using the Next Linear Collider Test Accelerator (NLCTA) beam line at the SLAC National Accelerator Laboratory. During all irradiations, adherent cells were maintained in a 37oC and 20% O2/5% CO2 environment. Clonogenic assays were performed following irradiation by seeding petri dishes at low cell densities and staining with 0.25% crystal violet. Colonies were counted using Matlab software to determine changes in cell survival due to both time of dose delivery and beam quality, and the survival data were fitted with the linear-quadratic model. DNA microarrays were performed to profile differences in gene expression between cells irradiated at the same dose over varying times.

Results: Cell lines varied in radiosensitivity, ranging from two to four logs of cell kill at the highest dose for both conventional and very rapid irradiation. Delivering radiation in shorter times decreased survival in all cell lines, indicating the radiobiologic advantage of faster radiation delivery. Log differences in cell kill ranged from 0.2 to 0.7 at 10 Gy for shorter irradiation compared to longer times for the studied cell lines. In addition, dose ratios at 5% survival varied from 1.03 to 1.08. Cell kill differences between short and long irradiation times were more pronounced as doses increased for all cell lines with statistical significance ($p < 0.05$) at higher doses.

Conclusions: Our findings suggest that shortening delivery of therapeutic radiation doses to under 1 minute may improve tumor cell kill. This study demonstrates the potential advantage of technologies under development to deliver stereotactic ablative radiation doses very rapidly. The ability to administer RT at sub-second timescales could revolutionize patient therapy by both freezing physiologic motion and enhancing tumor cell killing.

Conclusion:

We have successfully accomplished specific aim 1 of the proposed study and are currently analyzing the in vivo experiments. The delay was caused by delays at the VHEE beam line at NLCTA.

Appendices:

Figure 1. Experimental setup for cell irradiations using 6 MV photon beams.

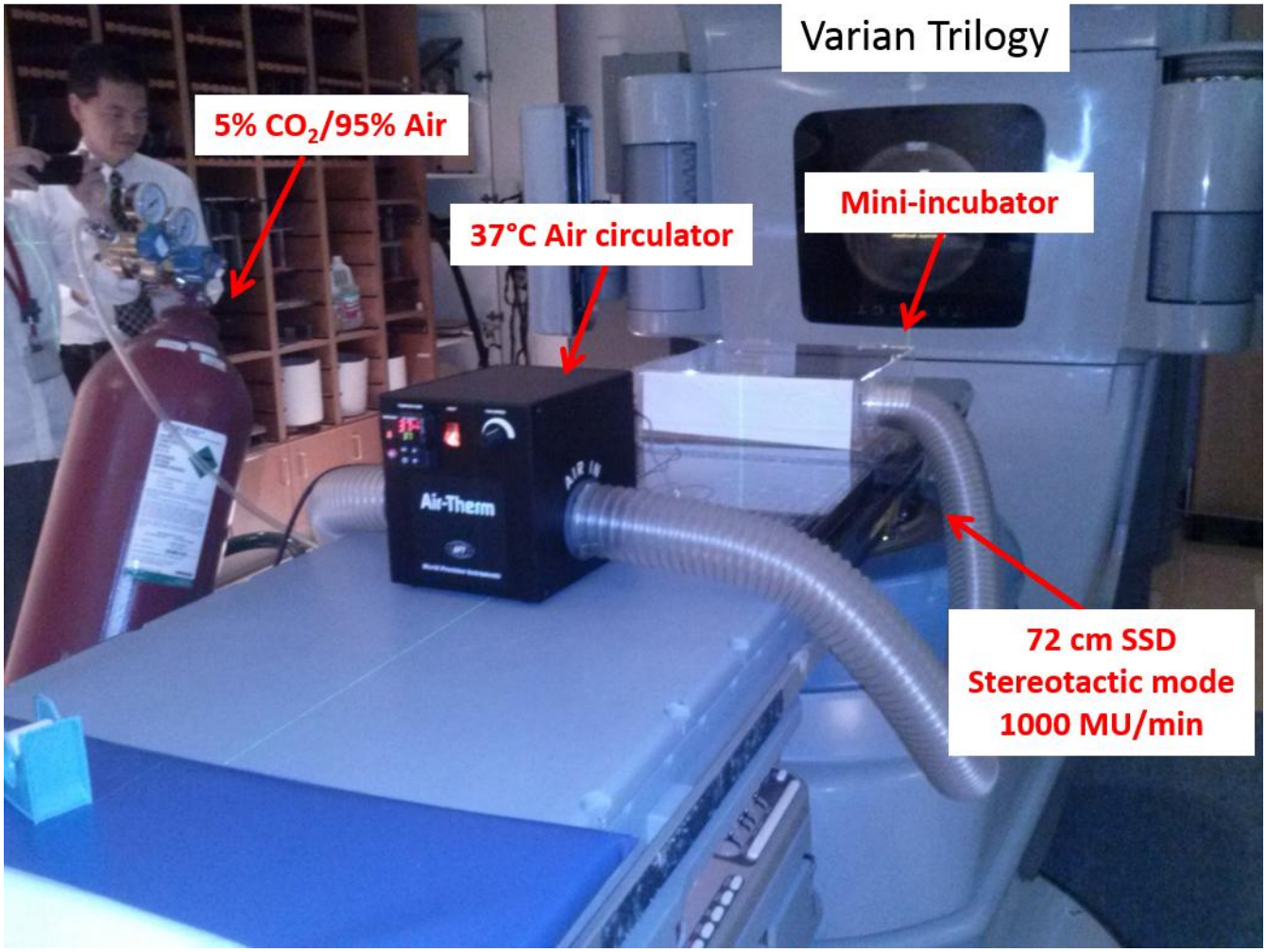


Figure 2. Experimental setup for cell irradiations using 9 MeV electron beams in total skin mode.

Figure 3. Example of colony formation in HCT116 cells.

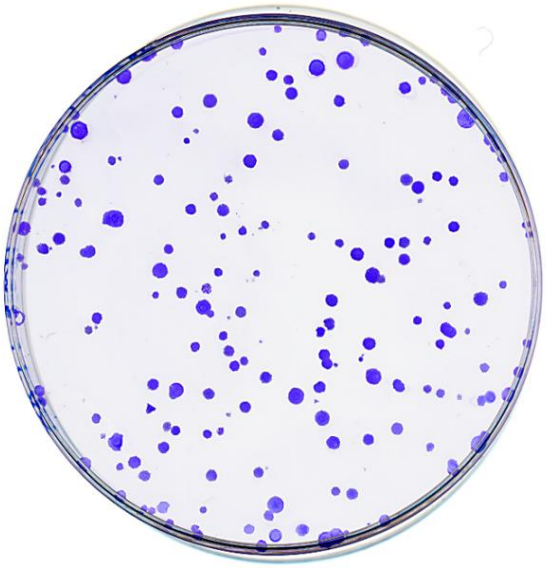


Figure 4. a) Experimental setup for the ***in vivo*** experiments using 10 MV photon beams from a clinical VARIAN TRUBEAM LINAC. b) Example of the kV images acquired before the irradiations. c) Enlargement of image a) to show the fiducial marker and the radiochromic film employed for dosimetry purposes.

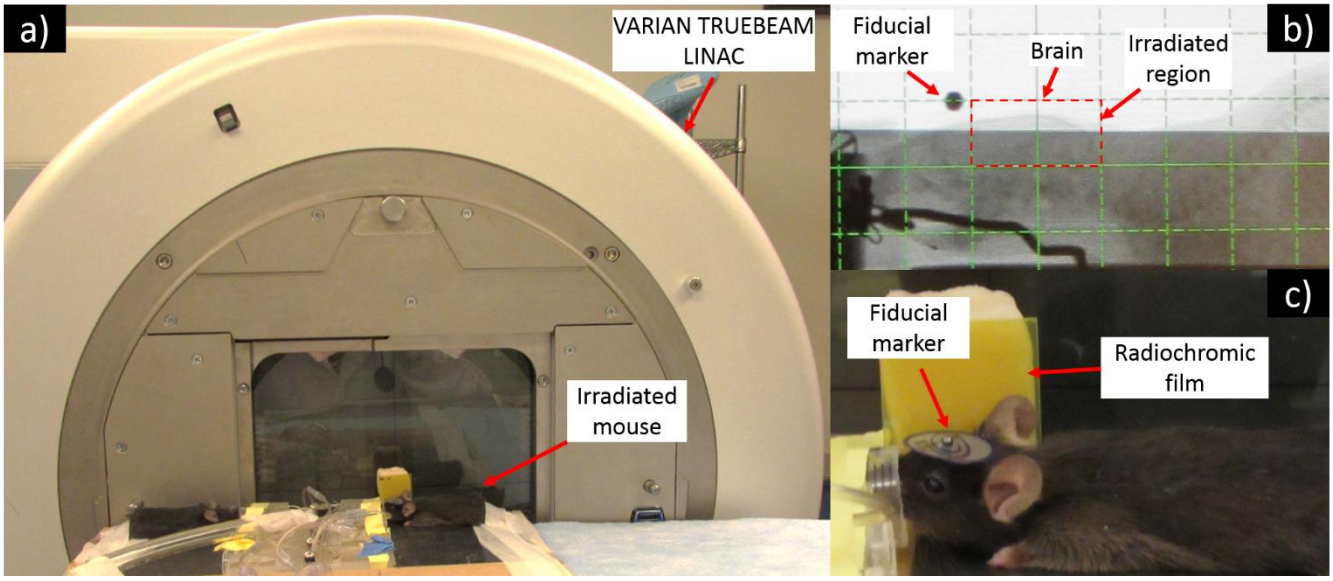


Figure 5. Experimental setup for the high energy electron irradiations. a) NLCTA beam line at SLAC National Laboratory. b) Shows the stage placed in front of the beamline with the mini-incubator on top of it. c) Mini-incubator containing the rotation motor that allowed the flask's two sided irradiation.

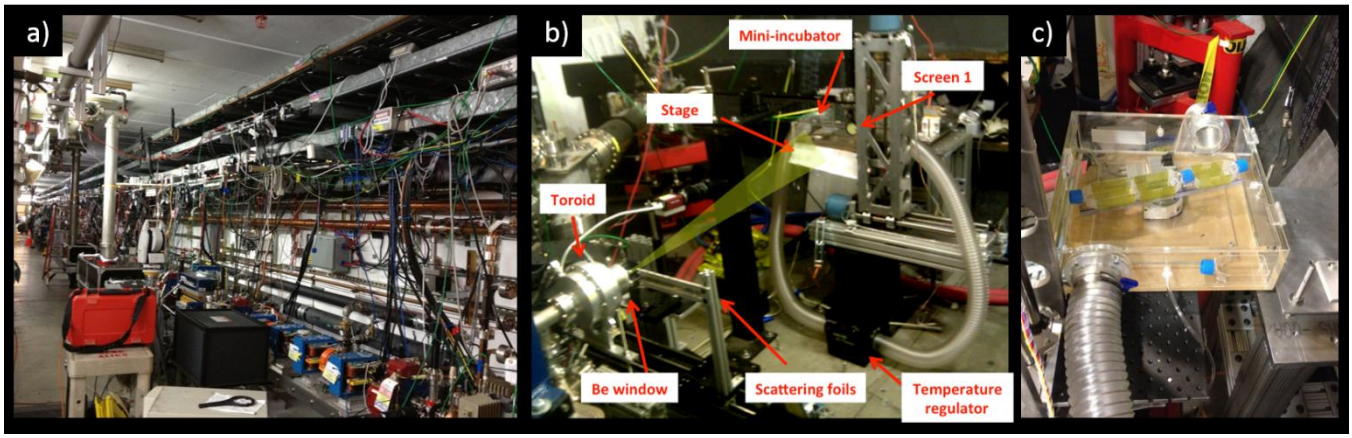


Figure 6. Example of the dose maps and profiles that were obtained from the dosimetry perfomed for the high energy electron irradiations.

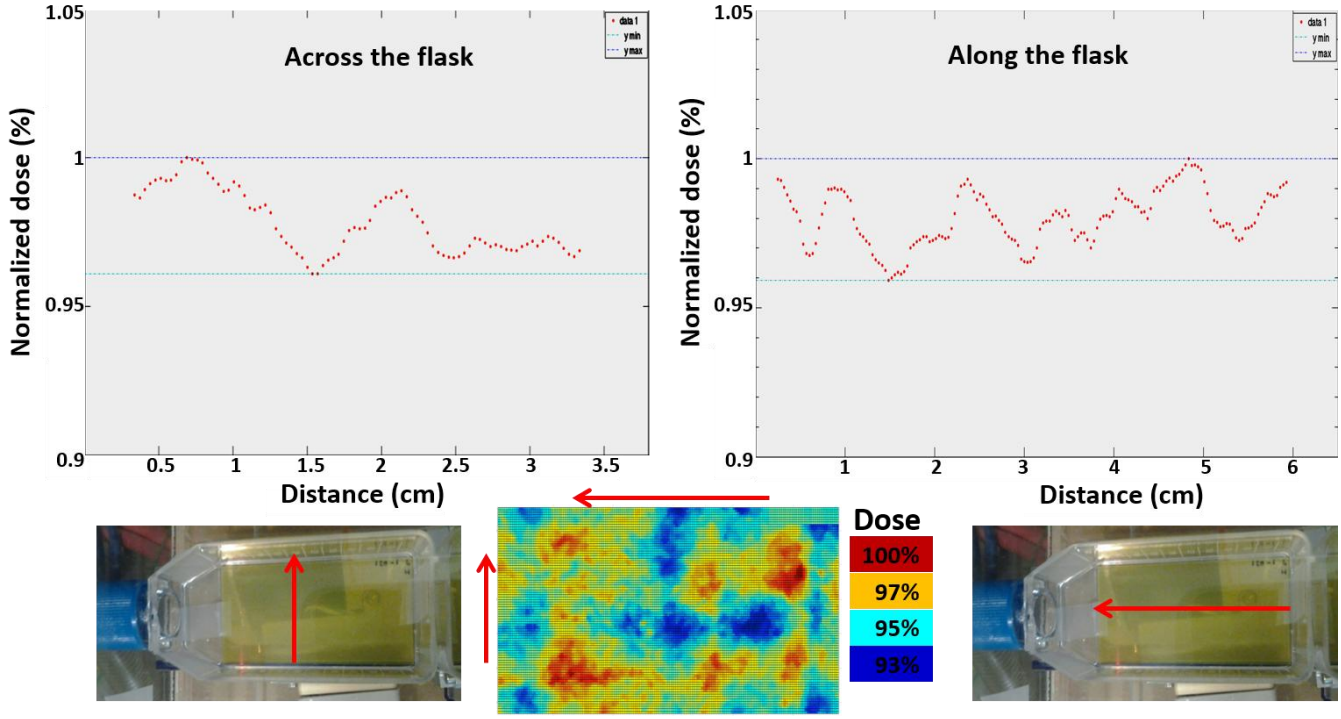


Figure 7. Computed tomography (CT) images of a mouse used to determine the target position from anatomical landmarks.

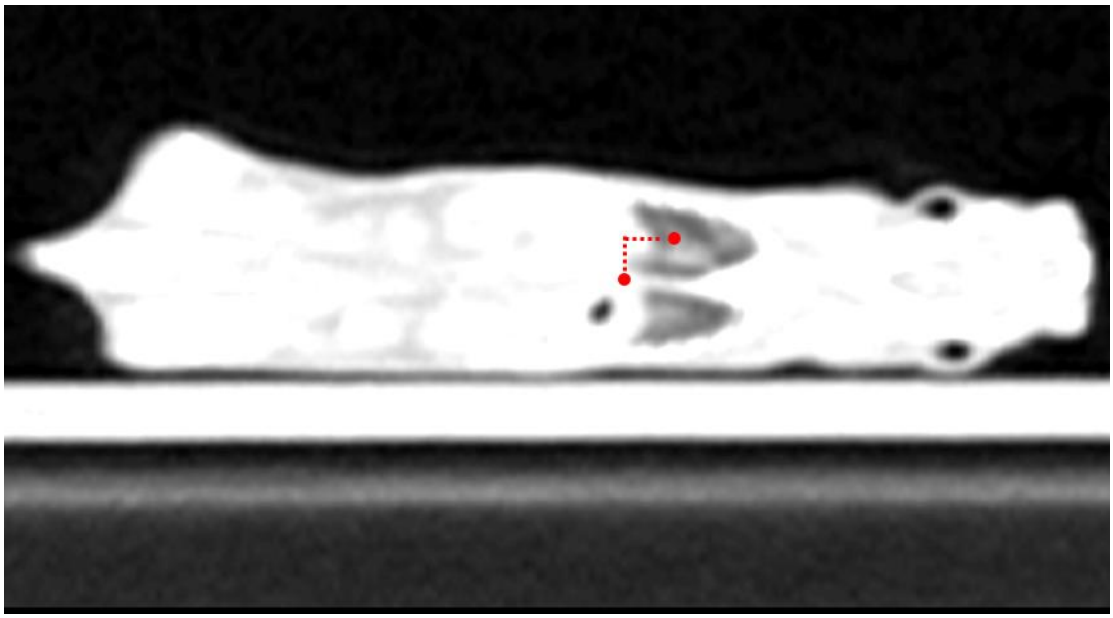


Figure 8. Experimental setup for the lung irradiation of mice using experimental VHEE at NLCTA.

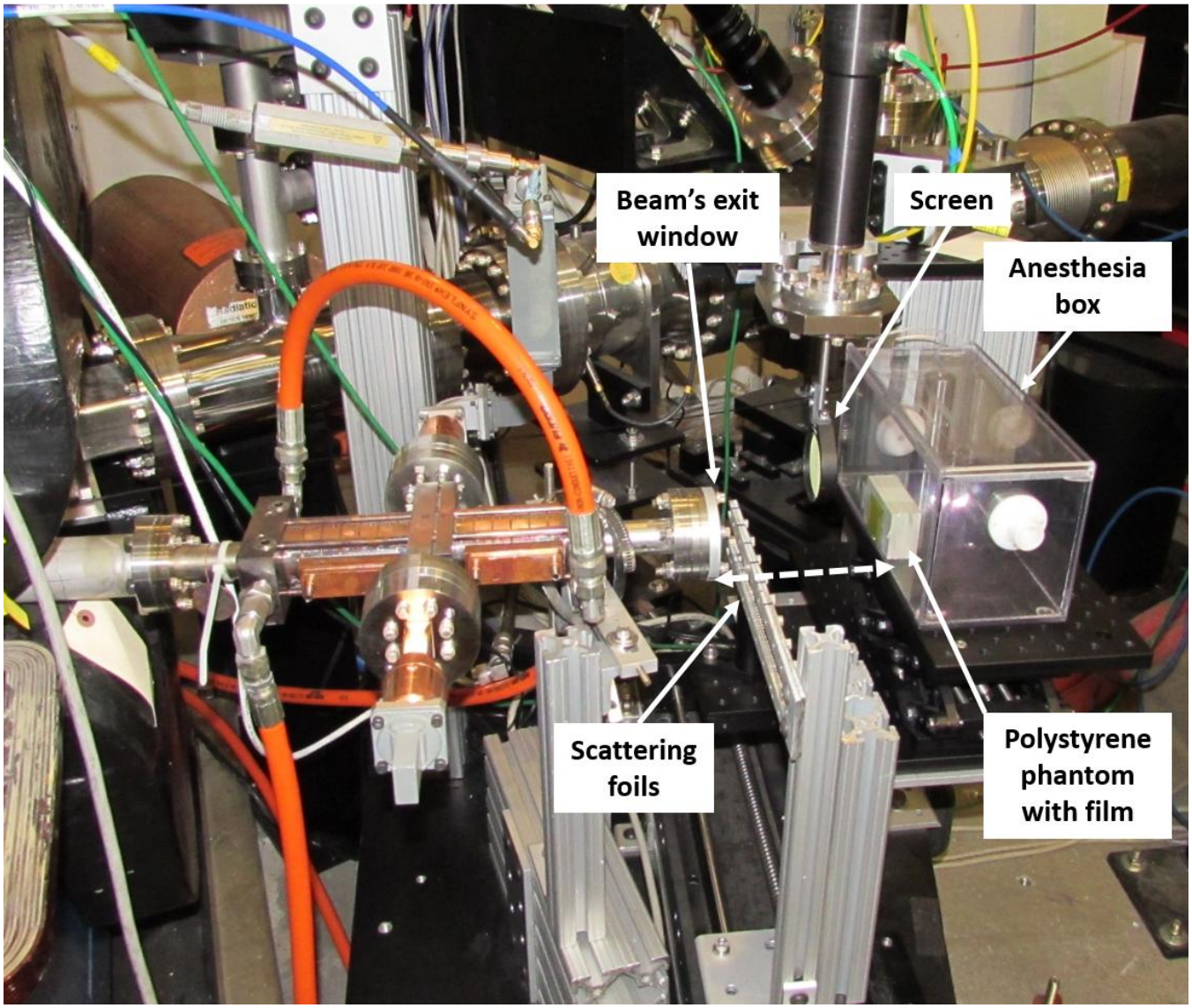


Figure 9. Experimental setup for the lung irradiation of mice using VHEE at NLCTA.

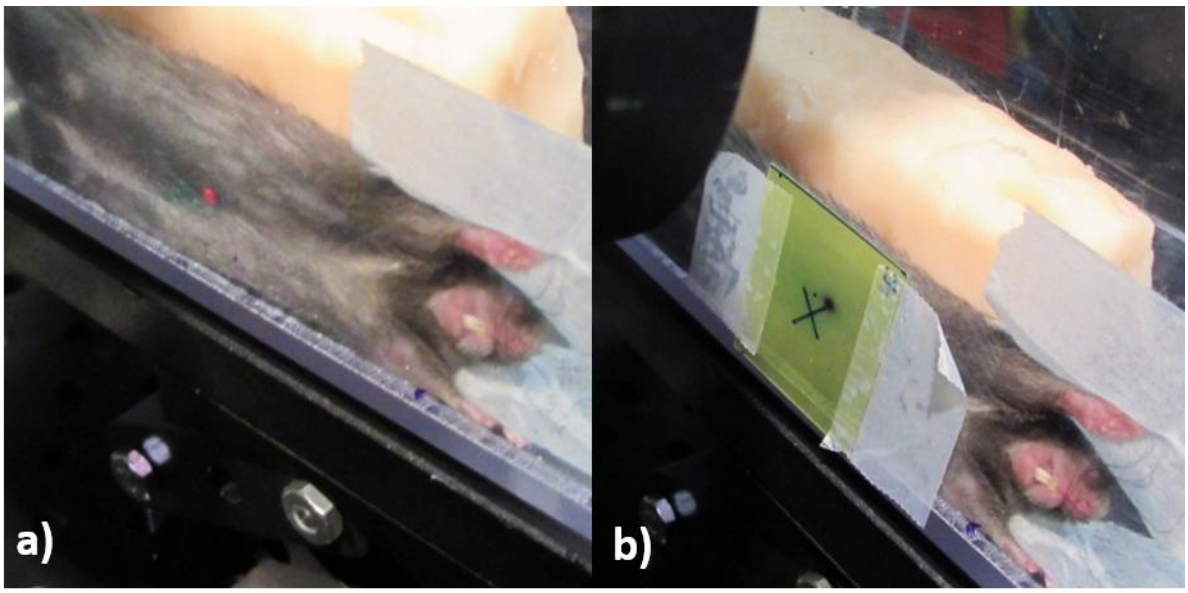
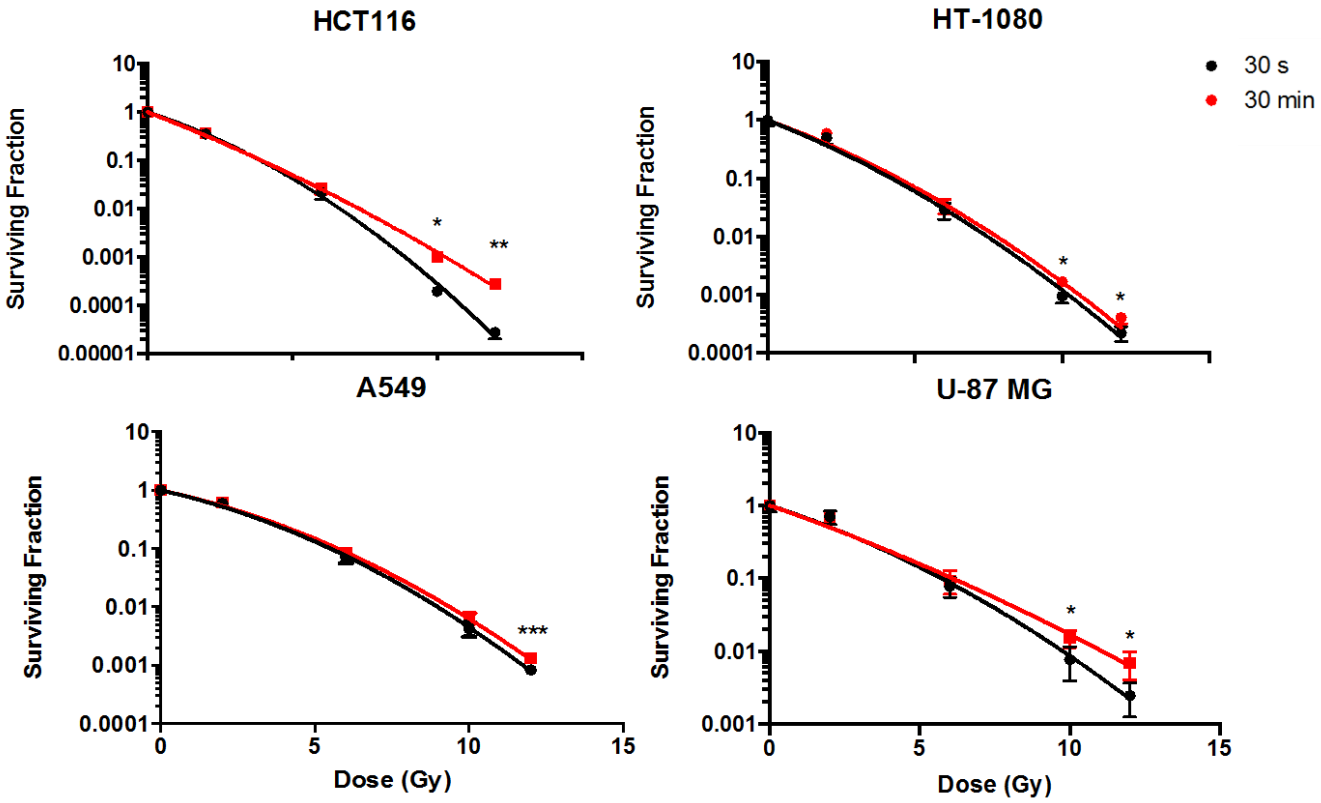
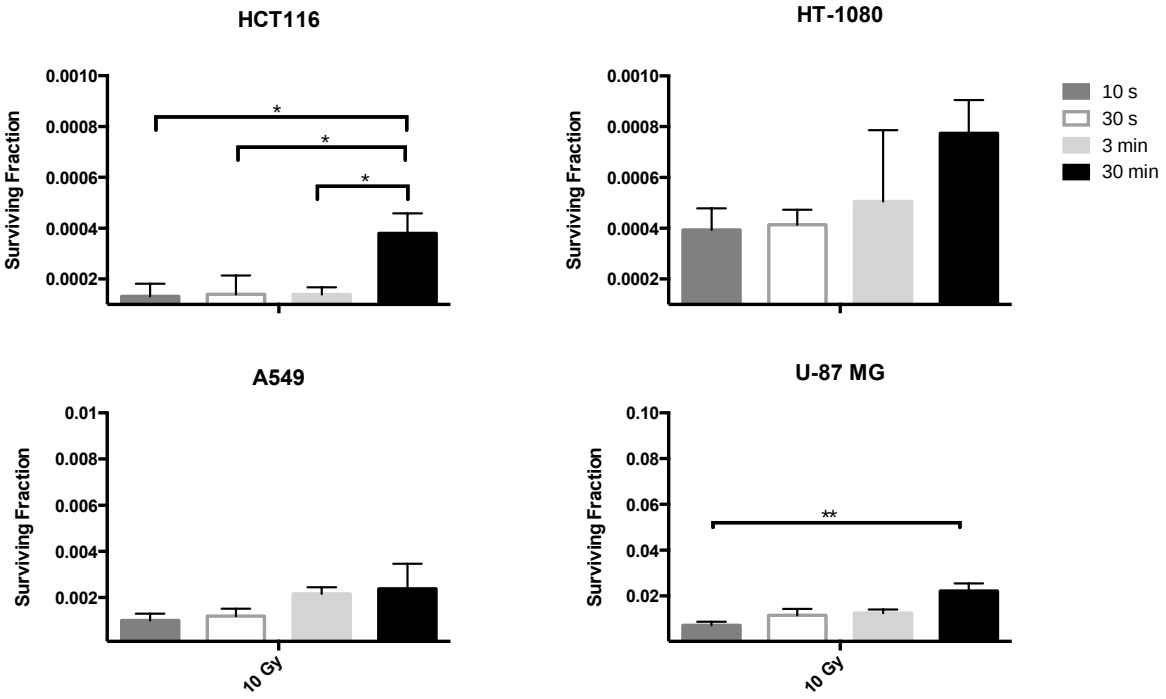


Figure 10. Survival curves corresponding to photon irradiations.



*p < 0.05, **p<0.01, ***p<0.001

Figure 11. Clonogenic survival analysis corresponding to electron irradiations The delivered dose was 10 Gy and the irradiation times were varied from 10s, 30s, 3 min, and 30 min.



***p < 0.05, **p < 0.01**

Figure 12. Survival curves obtained from the VHEE irradiations.